Endothelial lipase releases saturated and unsaturated fatty acids of high density lipoprotein phosphatidylcholine

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Abstract **We assessed the ability of endothelial lipase (EL) to hydrolyze the *sn*-1 and *sn*-2 fatty acids (FAs) from HDL phosphatidylcholine. For this purpose, reconstituted discoidal HDLs (rHDLs) that contained free cholesterol, apolipoprotein A-I, and either 1-palmitoyl-2-oleoylphosphatidylcholine, 1-palmitoyl-2-linoleoylphosphatidylcholine, or 1-palmitoyl-2-arachidonylphosphatidylcholine were incubated with EL- and control (LacZ)-conditioned media. Gas chromatography analysis of the reaction mixtures revealed that both the *sn*-1 (16:0) and *sn*-2 (18:1, 18:2, and 20:4) FAs were liberated by EL. The higher rate of *sn*-1 FA cleavage compared with *sn*-2 FA release generated corresponding *sn*-2 acyl lyso-species as determined by MS analysis. EL failed to release *sn*-2 FA from rHDLs containing 1-*O*-1′-hexadecenyl-2-arachidonoylphosphatidylcholine, whose *sn*-1 position contained a nonhydrolyzable alkyl ether linkage. The lack of phospholipase A_2 activity of EL and its ability to liberate [^{14}C]FA from [^{14}C]lysophosphatidylcholine (lyso-PC) led us to conclude that EL-mediated deacylation of phosphatidylcholine (PC) is initiated at the *sn*-1 position, followed by the release of the remaining FA from the lyso-PC intermediate. Thin-layer chromatography analysis of cellular lipids obtained from EL-overexpressing cells revealed a pronounced accumulation of [^{14}C]phospholipid and [^{14}C]triglyceride upon incubation with 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC-labeled HDL_3, indicating the ability of EL to supply cells with unsaturated FAs.**—Gauster, M, G. Rechberger, A. Sovic, G. Hörl, E. Steyrer, W. Sattler, and S. Frank. **Endothelial lipase releases saturated and unsaturated fatty acids of high density lipoprotein phosphatidylcholine. *J. Lipid Res.* 2005.** 46: **1517–1525.**

Supplementary key words phospholipases • lysophospholipases • phospholipids • polyunsaturated fatty acid • reconstituted high density lipoprotein

Endothelial lipase (EL) is a member of the triglyceride (TG) lipase gene family synthesized by various tissues and cell types, including endothelial cells. Endothelial expression is a distinct feature of this lipase compared with other family members (1, 2). EL is a phospholipase with a low but detectable TG lipase activity (1, 2), whereby the HDL phospholipids (PLs) are a preferred substrate for EL (3). The EL-mediated depletion of HDL-PL alters the composition and physical properties of HDL, resulting in a diminished ability of HDL to promote scavenger receptor class B type I-mediated [^{3}H]cholesterol efflux (4). Independent of its phospholipase activity, EL facilitates, by virtue of its bridging function, HDL particle binding and uptake (5, 6), as well as selective uptake of HDL cholesteryl esters (5). Experiments in mice with the disrupted native EL locus (7, 8) as well as in transgenic mice harboring the human EL gene (LIPG locus) (7), revealed an inverse relationship between plasma HDL cholesterol level and EL expression. Most recently, EL was found to facilitate the progression of atherosclerosis in apolipoprotein E (apoE)-deficient mice (9).

Initial studies on EL activity, using phosphatidylcholine (PC) labeled in the *sn*-1 position, established phospholipase A_1 (PLA_1) activity of the enzyme (2). Accordingly, *sn*-1 fatty acids (FAs) from HDL phosphatidylcholine (HDL-PC), the most abundant HDL-PL (10), are released by EL. The ability of EL to release [^{14}C]arachidonic acid from the 1-stearoyl-2-[1-^{14}C]arachidonyl PC-labeled HDL (11) suggested *sn*-2 phospholipase activity of EL. Furthermore, we found a decreased amount of the fatty acid synthase (FAS) mRNA and a decreased rate of fatty acid synthesis in EL-overexpressing cells (12). This was most likely a con-

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EL, endothelial lipase; EL-Ad, adenovirus encoding human endothelial lipase; FA, fatty acid; FCS, fetal calf serum; FFA, free fatty acid; GC, gas chromatography; HDL-PC, HDL phosphatidylcholine; LacZ-Ad, adenovirus encoding β-galactosidase; lyso-PC, lysophosphatidylcholine; MOI, multiplicity of infection; PAPC, 1-palmitoyl-2-arachidonylphosphatidylcholine; PL, phospholipid; plasmalogen-PC, 1-*O*-1′-hexadecenyl-2-arachidonoylphosphatidylcholine; PLPC, 1-palmitoyl-2-linoleoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; TG, triglyceride; TLC, thin-layer chromatography.

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Manuscript received 10 February 2005 and in revised form 30 March 2005.
Published, JLR Papers in Press, April 16, 2005.
DOI 10.1194/jlr.M500054-JLR200

sequence of the suppressing effect of polyunsaturated fatty acids on the expression of cellular lipogenic genes, including FAS (13).

In this report, we provide evidence for the ability of EL to liberate saturated as well as unsaturated FAs from reconstituted discoidal HDL (rHDL) and HDL_3-PC. We found that the mechanism of unsaturated FA release does not involve *sn*-2 phospholipase activity. Instead, the release of unsaturated FA requires two enzyme activities of EL, *sn*-1 phospholipase activity and lysophospholipase activity. Furthermore, we demonstrated the ability of EL to supply cells with unsaturated *sn*-2 FAs.

EXPERIMENTAL PROCEDURES

Preparation of EL- and LacZ-conditioned media

COS7 cells (1×10^6) were plated onto 100 mm dishes and incubated under standard conditions (37°C, 5% CO_2, and 95% humidity) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). After 24 h, cells were washed twice with phosphate-buffered saline (PBS) (pH 7.4) and infected with multiplicity of infection (MOI) 200 of adenovirus encoding human EL (EL-Ad) or adenovirus encoding β-galactosidase (LacZ-Ad) (4, 5) in DMEM without FCS. After 2 h, infection medium was replaced with serum-free medium (Panserin 401; Pan Biotech, Aidenbach, Germany) that was incubated with cells for 24 h. Prior to collection of the conditioned media, 10 U/ml heparin was added onto plates for 30 min. Thereafter, the media were collected into prechilled tubes and spun for 10 min in the bench centrifuge at 4°C to remove cell debris. Aliquots were kept at −70°C until used. A preparation of EL that generated 200 nmol of free fatty acid (FFA) (ml of EL)$^{-1}$ h^{-1} was used for subsequent experiments. Activity was determined by measuring the release of FFA from HDL_3 using a commercial kit (NEFA-C; Wako, Neuss, Germany) as described (4).

Isolation of human HDL

HDL (subclass 3, d = 1.125–1.21 g/ml) was prepared by sequential ultracentrifugation of plasma obtained from normolipidemic blood donors as described previously (5, 12). After ultracentrifugation, HDL was dialyzed against PBS (pH 7.4), and the protein concentration was determined by the Lowry method (14).

Preparation of rHDL

Discoidal rHDL containing either 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-linoleoylphosphatidylcholine (PLPC), 1-palmitoyl-2-arachidonylphosphatidylcholine (PAPC), plasmalogen-PC [1-*O*-1′ (Z)-hexadecenyl-2-arachidonylphosphatidylcholine] (Avanti Polar Lipids), free cholesterol (FC) (Sigma-Aldrich, St. Louis, MO) and apoA-I (kindly provided by Dr. G. M. Kostner) were prepared as described previously (15). Briefly, 3.29 μmol of the corresponding phospholipid was mixed with 13.9 nmol of FC, followed by the removal of chloroform by evaporation under a stream of argon. To the resulting film, 200 μl of Na-cholate (10% Na-cholate in 0.2 M potassium phosphate buffer, pH 7.4) was added dropwise with vortexing to give a clear solution, followed by the dropwise addition with vortexing of 559.2 μl of apoA-I (3.29 nmol). The resulting solution was extensively dialyzed against degassed PBS under argon at 4°C. The rHDLs were freshly prepared for each experiment, stored under argon at 4°C, and used within 3 days.

Analysis of FFAs released from POPC-, PLPC-, PAPC-rHDL, and HDL_3 by gas chromatography

To analyze FFAs liberated from rHDL-PL by EL, 350 μg rHDL (protein) was incubated with EL-conditioned medium (850 μl) and FFA-free BSA (Sigma) (50 μl) prepared as a stock solution (100 mg/ml) in Panserin 401 medium in a water bath at 37°C for 20 h. For the analyses of FFAs liberated from HDL_3, 500 μg HDL_3 (protein) was incubated with 446 μl EL-conditioned medium in the presence of FFA-free BSA (1%) for 20 h at 37°C. In the control incubations, EL-conditioned medium was replaced with LacZ-conditioned medium. Subsequently, incubation mixtures were split and one aliquot was extracted twice with hexane-isopropanol (3:2; v/v) containing glacial acetic acid (0.01%; v/v), evaporated under N_2, redisolved in chloroform, and loaded onto thin-layer chromatography (TLC) plates. The TLC separation of lipids was performed under argon using hexane-diethylether-glacial acetic acid (70:29:1; v/v/v) as the mobile phase. The lane loaded with oleic acid as a marker for the FFA migration was cut off the TLC plate, exposed to I_2 vapor, and used to determine the zones corresponding to FFA. The FFAs were scraped from the plates, followed by transesterification and gas chromatographic analysis as described (16). Briefly, 10 μg pentadecanoic acid as an internal standard was added to the scraped material, and transesterification was performed in the presence of 1 ml toluene and 1 ml boron trifluoride-methanol (20%) at 110°C for 60 min. Excess boron trifluoride was destroyed by the addition of 2 ml water, and the fatty acid methyl esters were extracted into 200 μl of hexane (final volume of the organic phase containing hexane and toluene was ~1.7 ml). Two microliters were analyzed by gas chromatography (GC). Separation of fatty acid methyl esters was performed on a CP-FFAP CB column (25 m, 0.32 mm id) using an Hewlett Packard (HP) 5890 gas chromatograph (CHROMPACK, Palo Alto, CA) equipped with a flame-ionization detector and a split/splitless injector. Helium was used as carrier gas; the split ratio was ~10:1. The initial temperature, at 150°C, was programmed to 215°C at 2.5°C/min with a hold at 215°C for 10 min, then programmed to 230°C at 10°C/min with a hold at this temperature for 12.5 min. The detector temperature was 300°C, and the injector temperature was 300°C. Concentrations of fatty acids were calculated by peak area comparison with an internal standard.

Analysis of PC and lyso-PC in EL and LacZ incubation mixtures using POPC-, PLPC-, PAPC-rHDL, and HDL_3 as substrates by tandem MS

For MS analysis, aliquots of the EL and LacZ incubation mixtures used for the analysis of FFA (see above) were extracted according to the method of Bligh and Dyer (17). Dried samples were redissolved with chloroform-methanol (2:1; v/v) and mixed with an equal volume of the internal standard solution [8.8 mg 1,2-distearoyl-D70-3-*sn*-glycerophosphatidylcholine in 10 ml chloroform-methanol (2:1; v/v)]. The mixture was diluted 1:100 with chloroform-methanol (2:1; v/v), and 20 μl of the diluted sample was mixed with 20 μl ammonium hydrogen carbonate solution and 160 μl chloroform-methanol (2:1; v/v). The mixture was used for MS analysis in a positive mode using the TSQ7000 triple quadrupole mass spectrometer (Finnigan-MAT; San Jose, CA) equipped with off-line nano-electrospray ion source (Protana; Odense, Denmark). PCs were detected by precursor ion scanning of *m/z* 184 in the positive ion mode with the following settings: spray voltage, 800 V; scan time, 400–1,200 *m/z* 4 s; collision energy, 35 eV. The scan was performed for 4 min, and ~60 tandem MS scans were averaged. The internal standard peak at *m/z* 860 was used to calculate a correction factor to compare the peak intensities of different samples (18).

Assay of *sn*-2-phospholipase activity

To assess whether EL exhibits PLA_2 activity, 150 μg rHDL containing plasmalogen-PC as the only PL was incubated with EL- and LacZ-conditioned medium in the presence of 1% FFA-free BSA at 37°C. As a positive control, rHDL containing plasmalogen-PC (150 μg) was incubated with 0.46 mU PLA_2 from porcine pancreas (Sigma) in PBS in the presence of 10 mM $CaCl_2$ and 1% FFA-free BSA. Aliquots (10 μl) were removed from the incubation mixtures at the indicated time points, and the release of FFA from plasmalogen-PC was determined using a commercial kit (NEFA-C; Wako Chemicals). After 20 h incubation, mixtures were extracted according to the method of Bligh and Dyer (17). The chloroform phase was evaporated under N_2, and lipids were redissolved with 50 μl of a chloroform-methanol (2:1; v/v) solution and applied on a TLC plate. Lipids were separated using chloroform-methanol-acetic acid-water (25:15:4:2; v/v/v/v) as the mobile phase and visualized by spreading 10% H_2SO_4 onto the plate, followed by heating at 120°C for 15 min.

Assay of lysophospholipase activity

The lysophosphatidylcholine (lyso-PC) substrate was prepared by mixing 1 μCi (18.18 nmol) of 1-[1-^{14}C]palmitoyl-2-lyso-PC (NEN; Boston, MA) with 901 μg (1.82 μmol) of unlabeled 1-palmitoyl-2-lyso-PC (Avanti Polar Lipids). The mixture was dried under a stream of N_2 and redissolved in 30 μl ethanol. Subsequently, lipids were solubilized in 970 μl DMEM by vortexing. For the time course experiment, aliquots (20 μl) of the lyso-PC mixture were mixed with 280 μl of EL- or LacZ-conditioned medium and incubated in the presence of 1% FFA-free BSA at 37°C for various periods of time, as indicated in the legend to Fig. 5A. Incubation mixtures were extracted twice with hexane-isopropanol (3:2; v/v) containing 1 N HCl, evaporated under N_2, redissolved in chloroform, and applied onto TLC plates. The TLC separation of lipids was performed using hexane-diethylether-glacial acetic acid (70:29:1; v/v/v) as the mobile phase. The TLC plate was exposed to I_2 vapor, and zones corresponding to FFA were cut out of the TLC plate and mixed with a scintillation cocktail, and radioactivity was determined on a β-counter (Beckman). For the dose-dependent study, increasing amounts of the lyso-PC mixture (5–60 μl) were incubated in a total volume of 280 μl of EL- or LacZ-conditioned medium at 37°C for 30 min. Samples were further processed as described for the time course experiment.

Lipoprotein labeling procedure

Labeling of HDL_3 with 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC (NEN) was performed as follows: 2 μCi of 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC was dried under nitrogen, redissolved in 30 μl ethanol, and added to a solution containing HDL_3 (3 mg protein) and lipoprotein-deficient serum (700 μl) in a final volume of 1.7 ml in PBS. Subsequently, this mixture was incubated under argon in a shaking water bath at 37°C. After 16 h incubation, labeled HDL_3 was reisolated by density gradient ultracentrifugation in a TLX120 bench-top ultracentrifuge in a TLA100.4 rotor (Beckman). The HDL_3 band was aspirated and desalted by size-exclusion chromatography using 10DG columns (BioRad). Specific activity obtained by this procedure was 1,900 cpm/μg HDL_3 protein.

Time course of in vitro EL-mediated hydrolysis of 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC-labeled HDL_3

1-Palmitoyl-2-[1-^{14}C]linoleoyl-PC-labeled HDL_3 (40 μg) was mixed with 425 μl of EL and LacZ incubation medium in the presence of 1% FFA-free BSA and incubated in a water bath at 37°C. At the indicated time points, incubation mixtures were extracted according to the method of Bligh and Dyer (17). Lipids were separated by TLC using chloroform-methanol-water (65:35:6; v/v/v) as the mobile phase, followed by exposure to a ^{14}C screen (Kodak) and visualization on the Storm imager (Amersham Biosciences). The signals corresponding to PC, lyso-PC, and FFA were cut out of the TLC plate, mixed with a scintillation cocktail, and measured on a β-counter.

TLC analysis of ^{14}C lipids in cell media and cells expressing EL, EL-Mut, and LacZ

HepG2 cells were plated onto 24-well trays and infected after 36 h with EL-, EL-Mut-, and LacZ-Ad at an MOI of 60. EL-Mut-Ad encodes catalytically inactive EL (5, 12). At 24 h postinfection, cells were washed twice with PBS and incubated with 300 μl DMEM containing the indicated concentrations of 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC-labeled HDL_3 and 2% FFA-free BSA under standard culture conditions. After 5 h, medium was collected and cells were incubated with DMEM containing 100 U/ml heparin to remove labeled HDL_3 bound to the cell surface. Subsequently, cells were extensively washed with PBS. The lipids were extracted twice from the media and cells using hexane-isopropanol (3:2, v/v), dried in the speed vac, and redissolved in chloroform before application onto the TLC plates. Hexane-diethylether-glacial acetic acid (70:29:1; v/v/v) was used as the mobile phase. Spots corresponding to PL, TG, and FFA were visualized upon exposure of the TLC plates to a ^{14}C screen (Kodak) on the Storm imager, cut out of the TLC plates, and measured by scintillation counting.

Statistics

Data are expressed as mean ± SD. Significance of differences was examined using Student's *t*-test.

RESULTS

EL releases both the *sn*-1 and *sn*-2 FAs from PC in rHDL

To examine the ability of EL to hydrolyze both the *sn*-1 and *sn*-2 FAs in PC, discoidal rHDL containing FC, apoA-I, and either POPC, PLPC, or PAPC were incubated with EL-conditioned medium obtained from COS7 cells infected with EL-Ad. Conditioned medium from LacZ-Ad-infected COS7 cells served as control. After 20 h incubation, the FA and lyso-PC concentrations were analyzed by GC or MS as described in the Experimental Procedures section. Incubation with EL-conditioned medium resulted in significantly higher concentrations of FAs liberated from the *sn*-1 and *sn*-2 positions, as compared with control LacZ incubations (**Fig. 1A**). Although EL was able to liberate *sn*-1 and *sn*-2 FAs, hydrolysis rates were higher for the *sn*-1 position for all PC subspecies used during these experiments (1.6-, 1.3-, and 1.6-fold; *sn*-1 vs. *sn*-2, respectively). With regard to FA specificity, 16:0-release from the *sn*-1 position was not significantly different between the PC species used, indicating that the *sn*-2 FAs do not interfere with the efficacy of *sn*-1 hydrolysis. In contrast, the FAs 18:1 and 18:2 of the *sn*-2 position appeared to be slightly preferred EL-substrates over FA 20:4. As expected from these results, MS analysis of lipids in the EL incubations revealed significantly higher concentrations of the corresponding *sn*-2 acyl-lyso-PC, as compared with *sn*-1 acyl-lyso-PC (Fig. 1B). The amount of the latter lyso-PC was similar in the EL and LacZ incubations, indicating that these subspecies were not generated by EL (Fig. 1B).

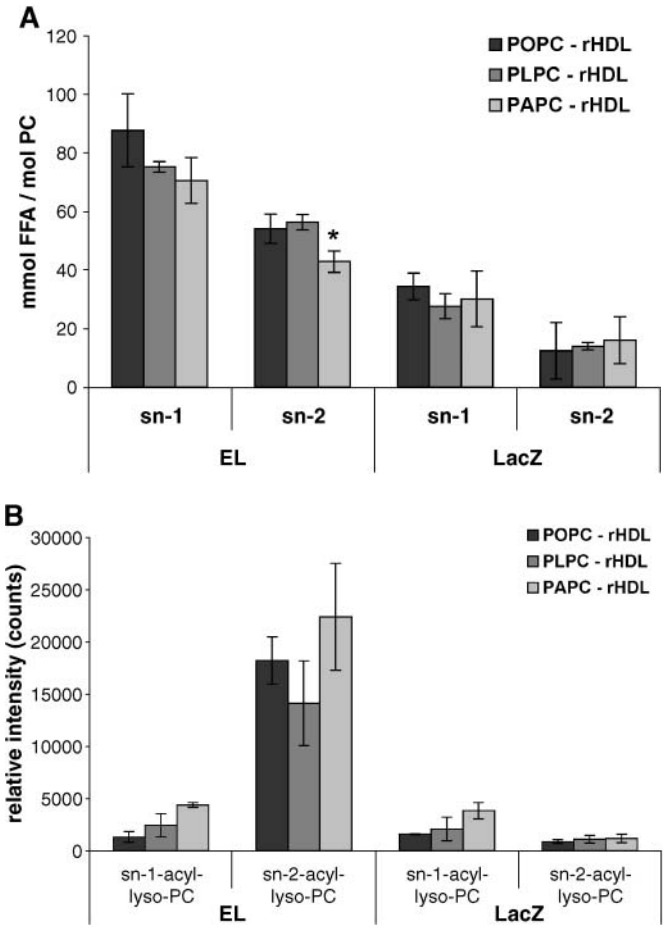

Fig. 1. Analysis of (A) free fatty acid (FFA) and (B) lysophosphatidylcholine (lyso-PC) released from reconstituted discoidal HDLs (rHDLs) by endothelial lipase (EL). A: rHDLs (350 μg protein) were incubated with 850 μl EL- or LacZ-conditioned medium in the presence of 1% FFA-free BSA at 37°C for 20 h. One aliquot of each incubation mixture was subsequently extracted twice with hexane-isopropanol (3:2; v/v) solution containing traces of glacial acetic acid (0.01%; v/v), and extracted lipids were separated on thin-layer chromatography (TLC) under argon using hexane-diethyl-ether-glacial acetic acid (70:29:1; v/v/v) as the mobile phase. The spots corresponding to FFA were scraped from the plates, followed by transesterification and gas chromatographic analysis, as described in Experimental Procedures. The amounts of endogenous FFA present in EL- and LacZ-conditioned media before incubation were subtracted from values obtained upon incubation. B: A second aliquot of the corresponding samples described in (A) was extracted according to the method of Bligh and Dyer (17) and analyzed by MS as described in Experimental Procedures. Results described in (A) and (B) are means ± SD of two independent experiments performed in triplicate. * $P \leq 0.05$ [compared with 1-palmitoyl-2-oleoylphosphatidylcholine (POPC)- and 1-palmitoyl-2-linoleoylphosphatidylcholine (PLPC)-rHDL].

TABLE 1. HDL_3-PC composition

PC	Total	SD	PC	Total	SD
	%			%	
PC 34:2	26.65	1.79	PC 32:0	0.73	0.04
PC 34:1	17.65	0.89	PC 40:6	0.64	0.05
PC 36:2	12.74	0.87	PC 38:1	0.51	0.08
PC 36:4	9.55	0.54	PC 40:5	0.45	0.04
PC 36:3	8.33	0.63	PC 32:2	0.35	0.03
PC 38:4	4.12	0.23	PC 40:4	0.29	0.04
PC 36:1	3.86	0.22	PC 30:0	0.24	0.05
PC 34:0	2.40	0.16	PC 40:7	0.21	0.02
PC 38:5	2.40	0.26	PC 30:2	0.17	0.09
PC 38:3	2.39	0.30	PC 40:2	0.12	0.01
PC 34:3	1.38	0.16	PC 40:3	0.10	0.04
PC 32:1	1.28	0.11	PC 28:1	0.09	0.02
PC 38:2	1.27	0.23	PC 40:1	0.07	0.01
PC 30:1	1.03	0.36	PC 40:0	0.06	0.01
PC 36:0	0.91	0.06	PC 28:0	0.04	0.01

PC, phosphatidylcholine. Values are means ± SD of a representative experiment performed in triplicate.

EL releases both saturated and unsaturated FAs from HDL-PC

Further experiments were performed to assess the ability of EL to liberate saturated and unsaturated FAs from its natural substrate, HDL-PC. First, we analyzed PC subspecies of HDL_3 lipids by MS. As shown in **Table 1**, PC 34:2 (16:0/18:2) was the most abundant PC subspecies, followed by PC 34:1 (16:0/18:1), PC 36:2 (18:0/18:2 or 18:1/18:1), PC 36:4 (16:0/20:4), PC 36:3 (18:1/18:2), PC 38:4 (18:0/20:4), and PC 36:1 (18:0/18:1). HDL_3 was then incubated with EL- and LacZ-conditioned media, and the incubation mixtures were analyzed by GC and MS, respectively, to determine the EL-mediated generation of FFA and lyso-PC. Importantly, these analyses demonstrated abundant FFA concentrations (**Fig. 2**) accompanied by increased concentrations of the corresponding lyso-PC compounds (**Table 2**). Consistent with its abundance in HDL-PC subspecies and preferential *sn*-1 localization, 16:0 was the most abundant FA in the EL incubation mixtures. The amounts of 18:0, 18:1, and 18:2 released from HDL-PC by EL were similar and ~2-fold lower compared with 16:0.

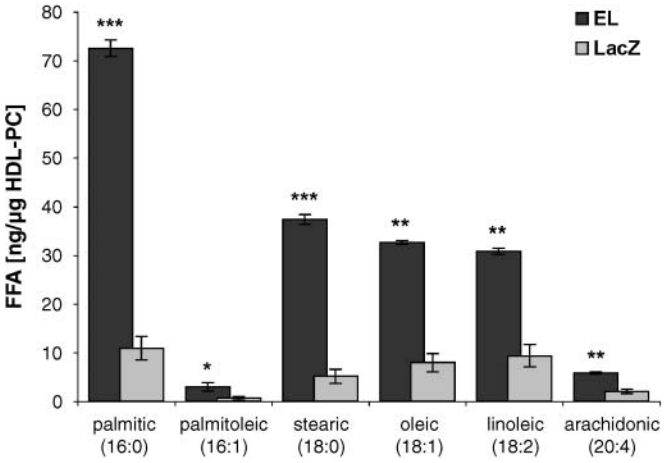

Fig. 2. Analysis of FFA liberated from HDL_3-PC by EL. HDL_3 was extracted according to the method of Bligh and Dyer (17), and lipids were analyzed by MS. HDL_3 (500 μg protein) was incubated with 446 μl of EL- and LacZ-conditioned media in the presence of FFA-free BSA (1%) at 37°C for 20 h. FFAs were determined as described in the legend to Fig. 1. Values are means ± SD of a representative experiment performed in triplicate. * $P \leq 0.05$ (compared with LacZ); ** $P \leq 0.01$ (compared with LacZ); *** $P \leq 0.001$ (compared with LacZ).

TABLE 2. Lyso-PC subspecies generated by the action of EL on HDL_3-PC

Lyso-PC	Total	SD
	%	
Lyso-PC 18:2	27.78	5.71
Lyso-PC 20:4	17.89	3.88
Lyso-PC 16:0	15.73	0.40
Lyso-PC 18:1	14.47	1.75
Lyso-PC 18:0	7.49	0.16
Lyso-PC 20:3	3.47	2.35
Lyso-PC 22:5	3.27	2.80
Lyso-PC 22:6	3.13	0.23
Lyso-PC 20:5	2.94	0.37
Lyso-PC 22:1	1.56	0.28
Lyso-PC 22:2	1.41	0.58
Lyso-PC 22:4	0.72	0.20
Lyso-PC 16:1	0.07	0.07
Lyso-PC 20:2	0.07	0.15

Values obtained in LacZ incubations were subtracted from EL incubations and are means ± SD of a representative experiment performed in triplicate.

The concentrations of 20:4 and 16:1 were 10- and 24-fold lower in the EL incubation mixture compared with 16:0 (Fig. 2). MS analyses of the incubation mixtures revealed a considerable EL-mediated increase in 18:2-lyso-PC, followed by 16:0-, 20:4-, 18:1-, and 18:0-lyso-PC, respectively (Fig. 2C). These results clearly demonstrated the ability of EL to liberate both saturated and unsaturated FAs from its natural substrate, HDL-PC.

Release of the *sn*-1 FA from PC by EL is a prerequisite for release of the *sn*-2 FA

To examine the mechanism responsible for the release of *sn*-2 FA from PC by EL, we first tested whether EL exhibits PLA_2 activity; i.e., we tested the ability of EL to release FA from the *sn*-2 position when the *sn*-1 position is occupied with *sn*-1 FA. For this purpose, apoA-I was reconstituted with plasmalogen (1-alkenyl-2-acyl-PC), a PC whose *sn*-1 position contained a nonhydrolyzable alkyl ether linkage. The *sn*-2 phospholipase activity was determined by measuring the FA release and the concomitant conversion of plasmalogen-PC into lyso-plasmalogen-PC. As shown in **Fig. 3A**, EL failed to liberate FA from plasmalogen-PC. Accordingly, plasmalogen-PC was not converted into lyso-plasmalogen-PC, as revealed by TLC analysis (Fig. 3B). In contrast, PLA_2, which serves as a positive control, liberated FAs very efficiently from plasmalogen-PC (Fig. 3A), resulting in a quantitative conversion into its lyso-plasmalogen-PC product (Fig. 3B). From these results, we concluded that EL lacks PLA_2 activity and that the EL-mediated release of the *sn*-1 FA must precede the release of the *sn*-2 FA.

If this model is correct, EL-mediated hydrolysis of 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC (P-L–PC) should result in the generation of a [^{14}C]linoleoyl-lyso-PC intermediate prior to the release of [^{14}C]linoleic acid. To test this assumption, HDL_3 was labeled with P-L–PC and incubated with EL and LacZ incubation media for various time points up to 5 h. TLC analysis of lipids recovered from EL incubations revealed an efficient and time-dependent hydrolysis of

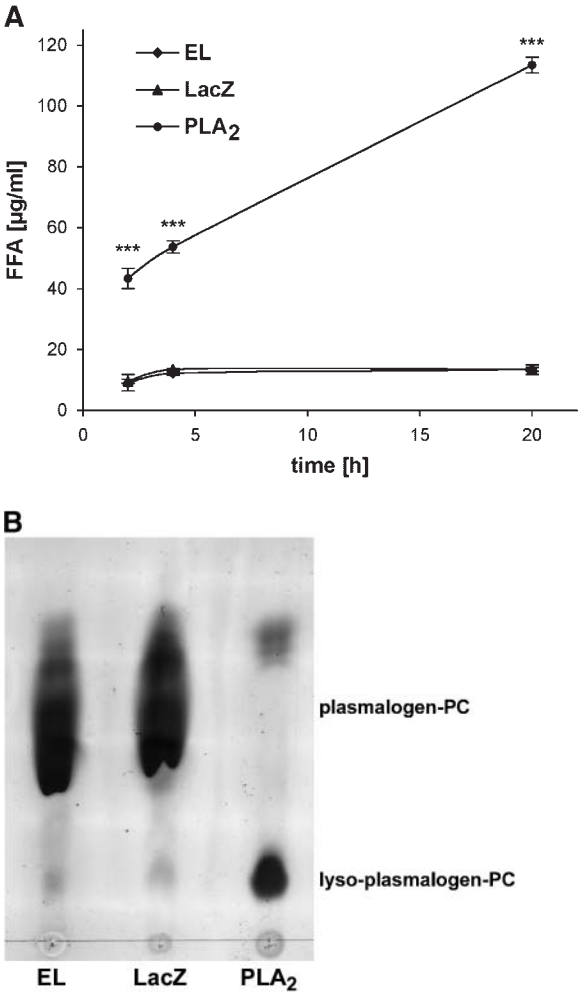

Fig. 3. Assessment of EL-*sn*-2-phospholipase activity. rHDL (150 µg protein) containing 1-*O*-1′-hexadecenyl-2-arachidonoylphosphatidylcholine as the only phospholipid (PL) was incubated with EL- and LacZ-conditioned media in the presence of 1% FFA-free BSA at 37°C. In parallel, rHDL was incubated with 0.46 mU phospholipase A_2 in PBS in the presence of 10 mM $CaCl_2$ and 1% FFA-free BSA. A: After the indicated periods of time, aliquots (10 µl) were removed from the incubation mixtures, and generation of FFA was determined using a commercial kit (Wako Chemicals). Results are means ± SD of a representative experiment performed in triplicate. *** $P \leq 0.001$, compared with EL and LacZ. B: After 20 h, lipids were extracted according to the method of Bligh and Dyer (17) and loaded onto TLC plates. Lipids were separated using chloroform-methanol-acetic acid-water (25:15:4:2; v/v/v/v) as the mobile phase and visualized by spreading 10% H_2SO_4 onto the plate followed by heating at 120°C for 15 min.

P-L–PC. A pronounced decrease in P-L–PC signal intensity was accompanied by a concomitant increase of [^{14}C]18:2-lyso-PC and of [^{14}C]18:2 signal intensities (**Fig. 4A**). In contrast, hydrolysis of HDL_3-associated P-L–PC by LacZ-conditioned medium was much less efficient (Fig. 4B). Scintillation counting of the corresponding lipids revealed a considerable EL-mediated decrease in P-L–PC, accompanied by a pronounced increase in [^{14}C]18:2-lyso-PC and by a moderate accumulation of [^{14}C]18:2 FFA after 1 h incubation (Fig. 4C). The pronounced increase in [^{14}C]18:2-lyso-PC clearly demonstrated that the EL-medi-

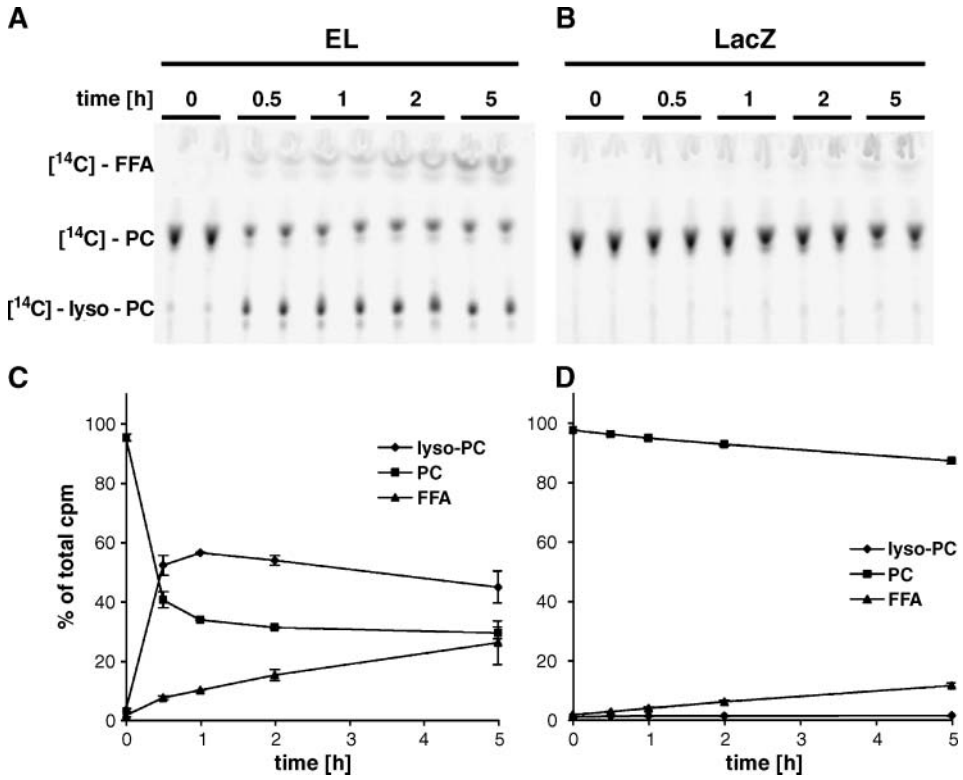

Fig. 4. Time course of EL-mediated hydrolyses of 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC-labeled HDL_3. 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC-labeled HDL_3 (40 μg) was mixed with 425 μl of EL and LacZ incubation medium in the presence of 1% NEFA-free BSA and incubated in a water bath at 37°C. After the indicated periods of time, incubation mixtures were extracted according to the method of Bligh and Dyer (17). A and B: Lipids were separated by TLC using as a mobile phase a mixture of chloroform-methanol-water (65:35:6; v/v/v) followed by exposure to a ^{14}C screen (Kodak) and visualization on the Storm imager. C and D: The signals corresponding to phosphatidylcholine (PC), lyso-PC, and FFA were measured by scintillation counting. Results are expressed as percent of total cpm, whereby total cpm for each incubation mixture represent the sum of [^{14}C]PC, -lyso-PC and -FFA signals. Results in (A) represent one experiment performed in duplicate. Results in (B) are means ± SD from two independent experiments performed in duplicate.

ated release of the *sn*-1 FA precedes the release of the *sn*-2 FA. During the next 4 h, the rate of P-L–PC hydrolysis declined and was accompanied by a decrease in [^{14}C]18:2-lyso-PC and a concomitant increase in [^{14}C]18:2 FFA. In the LacZ incubation, a 10% decrease in P-L–PC over a 5 h incubation was accompanied by an increase in [^{14}C]18:2 FFA, most probably mediated by endogenous PLA_2 activity (Fig. 4D). Because EL lacks *sn*-2 phospholipase activity, these results strongly suggest that [^{14}C]18:2 FAs were liberated by lysophospholipase activity of EL from [^{14}C]18:2-lyso-PC, generated as an intermediate by the action of EL *sn*-1-phospholipase activity on P-L–PC.

EL exhibits lysophospholipase activity

It is well established that under physiological conditions, FAs migrate spontaneously and rapidly from the *sn*-2 to the *sn*-1 position of lyso-PC, with an equilibrium ratio of 9:1 (19). Accordingly, it is very likely that *sn*-2-acyl-lyso-PC intermediates generated by the action of EL on PC are rapidly converted into *sn*-1-acyl-lyso-PC isomers. To test the ability of EL to hydrolyze *sn*-1-acyl-lyso-PC isomers, 1-[1-^{14}C] palmitoyl-2-lyso-PC was used as a substrate, and the release of [^{14}C]palmitic acid was monitored. As shown in **Fig. 5A, B**, incubation of [^{14}C]lyso-PC with EL-conditioned medium resulted in an efficient time- and dose-dependent liberation of [^{14}C]palmitic acid. In contrast, only minimal amounts of [^{14}C]palmitic acid were liberated in LacZ incubations. These results clearly demonstrated that EL has, in addition to phospholipase activity, lysophospholipase activity. The latter is responsible for the liberation of the *sn*-2-FAs from HDL-PC.

EL supplies cells with unsaturated *sn*-2 FAs

Next, we examined whether EL can supply cells with unsaturated, *sn*-2 FAs, released from HDL-PC. For this, HepG2 cells were infected with EL-Ad, EL-Mut-Ad [an adenovirus encoding catalytically inactive EL (5, 12)], and LacZ-Ad and incubated with increasing concentrations of P-L–PC-labeled HDL_3. TLC analyses of cell media after a 5 h incubation period revealed a pronounced dose-dependent accumulation of [^{14}C]18:2 FFA in the media of EL-Ad-infected cells (**Fig. 6A**). In the media of EL-Mut-Ad- and LacZ-Ad-infected cells, the amount of [^{14}C]18:2 FFA was 6-fold lower than in EL media. TLC analysis of cellular

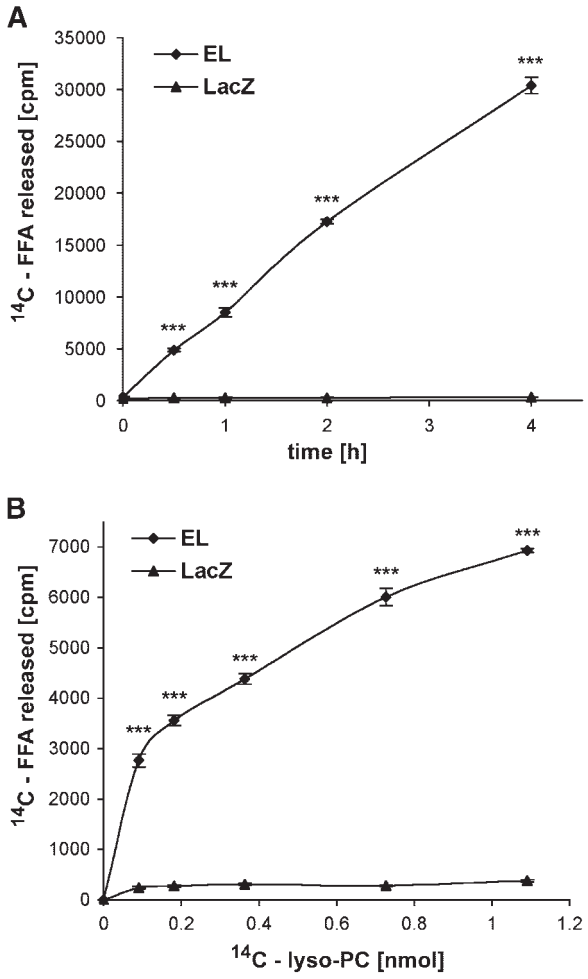

Fig. 5. Time-dependent (A) and dose-dependent (B) EL-mediated hydrolyses of [^{14}C]lyso-PC. A: A mixture of 1-[1-^{14}C]palmitoyl-*sn*-2-lyso-PC and unlabeled 1-palmitoyl-2-lyso-PC prepared as described in Experimental Procedures and containing 0.364 nmol [^{14}C]lyso-PC was incubated with 280 μl EL- or LacZ-conditioned medium in the presence of 1% FFA-free BSA at 37°C for the indicated periods of time. Incubation mixtures were extracted twice with hexane-isopropanol (3:2; v/v) containing 1 N HCl, evaporated under N_2, redisolved in chloroform, and applied onto TLC plates. The TLC separation of lipids was performed using hexane-diethylether-glacial acetic acid (70:29:1; v/v/v) as the mobile phase. The zones corresponding to FFA were measured by scintillation counting. B: Indicated amounts of [^{14}C]lyso-PC were incubated in a total volume of 280 μl EL- or LacZ-conditioned medium, in the presence of 1% FFA-free BSA at 37°C for 30 min. Samples were further processed as described in (A). Results (A) and (B) represent one representative experiment performed in triplicate. Results are means ± SD. *** $P \leq 0.001$ (compared with LacZ-conditioned medium).

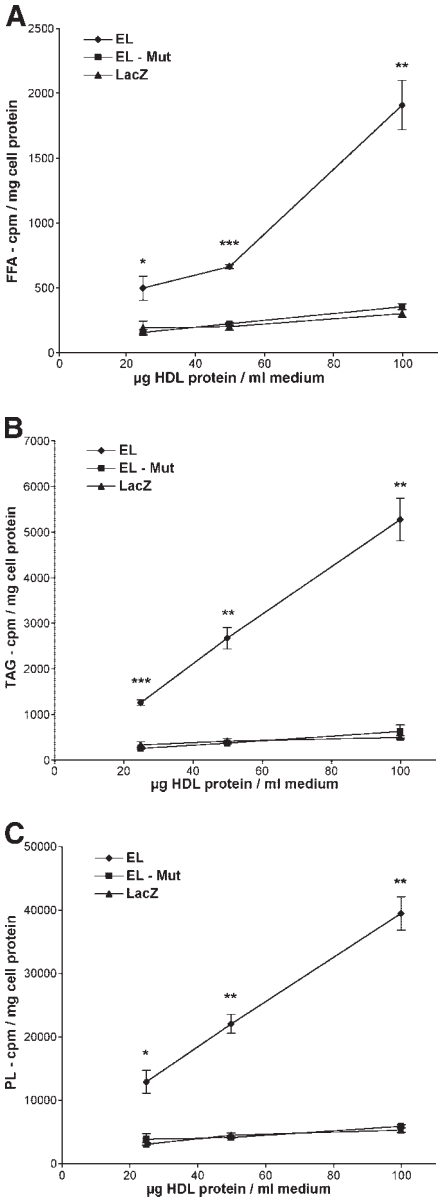

Fig. 6. Quantification of [^{14}C]FFA in cell media (A), [^{14}C]triglyceride (TAG) (B), and [^{14}C]PL (C), in cellular lipid extracts of HepG2 cells infected with adenovirus encoding human EL (EL-Ad), EL-Mut-Ad, and β-galactosidase (LacZ-Ad) after an incubation in the presence of 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC-labeled HDL_3. HepG2 cells were plated into 24-well trays and infected after 36 h with EL-, EL-Mut-, and LacZ-Ad at a multiplicity of infection of 60. At 24 h postinfection, cells were washed twice with PBS and incubated with 300 μl of Dulbecco's modified Eagle's medium (DMEM) containing the indicated concentrations of 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC-labeled HDL_3 and 2% FFA-free BSA under standard cell culture conditions. After 5 h, medium was collected and cells were incubated with DMEM containing 100 U/ml heparin, followed by three washes with PBS. The lipids were extracted twice from the media and cells with hexane-isopropanol (3:2; v/v), dried in a speed vac, and redissolved in chloroform prior to TLC separation. A mixture of hexane-diethylether-glacial acetic acid (70:29:1; v/v/v) was used as a mobile phase. The signals corresponding to PL, TG, and FFA were measured by scintillation counting. Results are means ± SD of a representative experiment performed in triplicate. *** $P \leq 0.001$ (compared with LacZ and EL-Mut); ** $P \leq 0.01$ (compared with LacZ and EL-Mut); * $P \leq 0.05$ (compared with LacZ and EL-Mut).

lipid extracts showed that EL-Ad-infected cells accumulated profoundly higher amounts of [^{14}C]TGs (Fig. 6B) and [^{14}C]PL (Fig. 6C) compared with EL-Mut-Ad- and LacZ-Ad-infected cells. These results clearly demonstrate that the interplay of both phospholipase and lysophospholipase activity in EL is a prerequisite for the supply of cells with unsaturated FAs from HDL-PC, which are, in turn, incorporated into endogenous lipids.

DISCUSSION

EL is a phospholipase with little TG lipase activity (1, 2). Previous studies have shown that EL efficiently liberates FA from HDL-PC, the preferential substrate of EL (3, 12). Results of the present study revealed the ability of EL to release saturated and unsaturated FAs from rHDL with defined PC composition as well as from native HDL_3. When POPC-, PLPC-, and PAPC-rHDLs were incubated with EL-conditioned medium, comparable amounts of the *sn*-1 FA (16:0) were hydrolyzed by EL from all three different substrates. Because PC substrates used in our study differed only in the length and saturation of their *sn*-2 FA, it appears that the cleavage efficiency of the *sn*-1 FA in PC is not affected by the *sn*-2 FA.

The results of the present study revealed that the rates of the EL-mediated release of the *sn*-2 FAs 18:1 and 18:2 were comparable but significantly exceeded that of the *sn*-2 FA 20:4. This effect was even more pronounced when HDL_3 was used as a substrate for EL. HDL_3 contained a 2.8-fold lower amount of the PC subspecies 36:4 (16:0/20:4) and 38:4 (18:0/20:4), both serving as sources of FA 20:4, compared with the PC subspecies 34:2 (16:0/18:2) and 36:2 (18:0/18:2) containing FA 18:2. Therefore, the 4.5-fold lower amount of FA 20:4 released by EL compared with FA 18:2 could not be explained by a low relative abundance of the PC subspecies supplying FA 20:4. Accordingly, it is tempting to speculate that the degree of unsaturation and the acyl chain length affect the cleavage efficiency of FA 20:4 from PC by EL.

We clearly demonstrated in the present study that EL lacks *sn*-2 phospholipase activity. However, like hepatic lipase (20, 21), EL has the ability to hydrolyze lyso-PC. Accordingly, the lower rate at which FA 20:4 is released from HDL-PC by EL, compared with the FAs 18:1 and 18:2, might be accounted for by a less-efficient EL-mediated hydrolyzation of 20:4-lyso-PC, compared with 18:1- and 18:2-lyso-PC. However, further experiments are required to clarify the impact of the acyl chain length and unsaturation on the rate of lyso-PC hydrolysis by EL.

In contrast to FAs 18:2 and 20:4, which reside on the *sn*-2 position of PC, 18:1 is distributed between both positions. For example, in PC 36:3, this FA resides on the *sn*-1 position; in PC 34:1, on the *sn*-2 position; and in PC 36:2 (18:0/18:2 or 18:1/18:1), on both positions. Accordingly, 18:1 released from HDL_3-PC by EL originates from both *sn* positions. Our analysis using GC revealed a strikingly high relative abundance of 16:0- and 18:0-lyso-PC in the EL-HDL_3 incubations, even at conditions of low relative abundance of the PC subspecies 32:0 and 36:0 in HDL_3. A possible explanation might be an efficient generation of these lyso-PC subspecies but their very inefficient cleavage by EL.

Experiments in which plasmalogen-PC, containing a nonhydrolyzable *sn*-1 position, was used as a substrate for EL have shown that EL completely lacks *sn*-2 phospholipase activity. From these results, we conclude that the initial EL-mediated deacylation occurs at the *sn*-1 position, resulting in a lyso-PC intermediate, which is, in turn, deacylated by the lysophospholipase activity of EL. Two lines of evidence support this assumption. First, a [^{14}C]linoleoyl-lyso-PC intermediate is formed upon the action of EL on 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC (P-L–PC) prior to the release of [^{14}C]linoleic acid. Second, EL has the ability to hydrolyze 1-[1-^{14}C]acyl-2-lyso-PC. It is well known that in aqueous medium at neutral pH, acyl chains rapidly migrate from the *sn*-2 to the deacylated *sn*-1 position to give a more stable intermediate (19). Therefore, it is very reasonable to assume such FA migration also under our experimental conditions. After an initial EL-mediated deacylation of HDL-PC, the remaining *sn*-2 acyl chain migrates to the *sn*-1 position, where it is, in turn, cleaved by the *sn*-1 phospholipase activity of EL. In light of these results, it is tempting to speculate that a less-efficient release of FA 20:4, compared with 18:1 and 18:2, from rHDL- or HDL_3-PC by EL might be independent of EL substrate preference and, in fact, could be due to a less-efficient migration of 20:4 to the *sn*-1 position for cleavage by EL-lysophospholipase activity.

Commercially available lyso-PC was a 9:1 mixture of *sn*-1- and *sn*-2-acyl-lyso isomers (22, 23). Therefore, although it is unlikely, we cannot rule out the possible contribution of the *sn*-2 lysophospholipase activity of EL. To address the regiospecificity of EL, we aimed at monitoring the rate of hydrolyses of *sn*-1- and *sn*-2 acyl-lyso-PC isomers by [^{31}P]NMR. This method requires high enzymatic activities in the studied enzyme, to promote rapid conversion of high amounts of substrate into a corresponding product (19). However, the relatively low abundance of EL in conditioned media, and the low yield of enzymatically active EL upon further purification, impeded [^{31}P]NMR analysis. Because acyl migration is inhibited at pH 5.5 (19), the ability of EL to release [^{14}C]FA from a 1-acyl-2-[1-^{14}C]acyl-PC at pH 5.5 would provide information on the regiospecificity of EL lysophospholipase activity. Unfortunately, at pH 5.5, both phospholipase and lysophospholipase activities of EL were nearly absent (not shown), precluding the possibility of addressing the regiospecificity of EL lysophospholipase activity by this approach.

Our previous study (12) indicated that FAs liberated by EL from HDL-PL are incorporated into endogenous lipids in EL-expressing cells. Because 1,2-di[1-^{14}C]palmitoyl-PC was used as a tracer in that study, we could not discriminate between [^{14}C]FAs released only from the *sn*-1 or *sn*-2 positions of HDL-PC. In the present study, using 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC-labeled HDL_3, we demonstrated the ability of EL to supply cells with *sn*-2 [^{14}C]FAs, which, in turn, are incorporated into cellular TG and PL. Markedly higher accumulation of [^{14}C]TG and [^{14}C]PL in EL-compared with EL-Mut-expressing cells excluded the possibility that the accumulation of the ^{14}C label in the cells is due to [^{14}C]HDL particle uptake and selective uptake of [^{14}C]HDL-PC, processes that might be mediated by the bridging function of EL (12, 24). A recent study demonstrated the ability of EL that is upregulated in the lipoprotein lipase (LPL)-deficient adipose tissue (AT) to supply that tissue with HDL-PC-derived FAs (25). Although EL only partially normalized FA import in adipocytes when LPL was absent, these in vivo results substantiate the role of EL in supplying cells and tissues with FAs derived from HDL-PC. Interestingly, LPL-deficient AT exhibited a highly significant reduction of FA 18:2 and a significant increase of FAs 16:1

and 18:1, respectively, in both TG and PL moiety. This finding is not in line with our results, demonstrating that amounts of FAs 18:1 and 18:2 released by EL from HDL-PC were similar and markedly higher compared with FA 16:1 (Fig. 2). This discrepancy might be due, at least in part, to the difference between human HDL_3 and mouse lipoproteins with regard to FA composition in their PC moiety.

Unsaturated FAs, notably 18:1 and 18:2, which can be supplied to cells by EL, have been shown to interfere with tumor necrosis factor-α (TNF-α)-induced expression of vascular adhesion molecule 1 (26, 27). Considering the fact that EL is upregulated by TNF-α (28), it is tempting to speculate that under inflammatory conditions, expression of adhesion molecules on vascular endothelium is, at least in part, affected by EL. Furthermore, considering the fact that EL has the ability to supply endothelial cells with HDL-derived arachidonic acid, which exerts a stimulatory effect on prostacyclin synthesis (29), EL might have an impact on vasorelaxation.

Taken together, our results indicate that EL has the ability to liberate both saturated and unsaturated FAs from HDL-PC because of its *sn*-1 phospholipase and lysophospholipase activity. Saturated and unsaturated FAs liberated from HDL-PC by EL are supplied to EL-expressing cells and are rapidly incorporated into endogenous lipids.

This work was supported by Grants SFB-F717 (S.F.) and SFB-F701 and 15.358 (E.S.) of the Austrian Science Funds (FWF); by Grant 10172 (S.F.) of the Austrian National Bank; by The Franz-Lanyar-Foundation, Grants 276 and 279 (S.F.); and by the Austrian Heart Foundation (E.S.). The authors appreciate the technical assistance of Helga Reicher, Birgit Reiter, Michaela Tritscher, and Katja Schick.

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